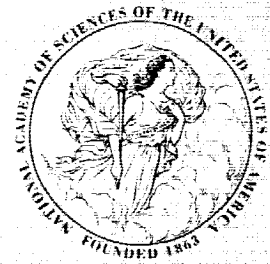# Proceedings

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Colony size distributions as a measure of *in vivo* and *in vitro* aging

(human diploid cells/cloning/*in vitro* life-span)

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ABSTRACT Individual human diploid cells plated at low cell density and incubated for 2 weeks develop into colonies ranging in size from one cell to several thousand cells. The resultant colony size distribution is an accurate indicator of the number of subsequent *in vitro* population doublings that can be attained by the parent culture. This relationship holds for both human fetal lung and adult skin fibroblast cultures. In addition, the colony size distributions obtained from fetal, young adult, and old adult human cell cultures at the same low level of *in vitro* passage are indicative of the *in vivo* age of the cell culture donor. Cell cultures of fetal origin give rise to the highest percentage of colonies with significant proliferative abilities, whereas cultures from old adults give rise to the lowest percentage of large colonies. Therefore, colony size distributions appear to be good indicators of both *in vitro* and *in vivo* human cellular aging.

A decline in cellular proliferative capacity may be an important aspect of *in vivo* mammalian aging (1–3). The ethical and practical limitations of *in vivo* human experimentation have led to a search for appropriate *in vitro* model systems to examine this diminished function. Cultured human diploid fetal lung fibroblasts, WI-38, have been extensively studied as a model because of their limited ability to proliferate *in vitro* (4). A number of other human cell types have subsequently been shown to have limited *in vitro* life-spans (5–7). Examination of cultures of adult human skin fibroblasts has revealed that their *in vitro* life-spans are inversely related to *in vivo* donor age (5, 6).

Recently it has been demonstrated that cell cultures from older human subjects, even during their first few *in vitro* passages, exhibit diminished replicative ability when compared to parallel cultures derived from young subjects (5). However, these studies have examined the replicative behavior of cell populations. Because human diploid cell cultures are heterogeneous in nature, it is also vital to examine the proliferative behavior of human cells as a function of aging at the level of the individual cell. Recent studies on human fetal lung cells (WI-38) have indicated that the distribution of sizes of colonies derived from single cells may be an excellent indicator of the total proliferative capacity (*in vitro* life-span) of human diploid cell cultures (8).

The studies described in this report compare the behavior of cloned human adult skin fibroblasts and fetal lung fibroblasts. In addition, the relationship between colony size distribution (CSD) and *in vivo* donor age is evaluated.

MATERIALS AND METHODS

Cell cultures were established from 2-mm skin biopsies performed on nonhospitalized volunteer members of the Baltimore Longitudinal Study as described (5). Cell cultures from old (>65 years) and young (20–35 years) donors were examined after 5–15 population doublings (PDs) *in vitro*.

The *in vitro* life-spans of these cell lines were determined at the Gerontology Research Center by described techniques (5). At low *in vitro* population doubling level (PDL), frozen stocks of these cultures were prepared by resuspending trypsinized cell suspensions in Eagle's minimal essential medium containing 10% fetal bovine serum and 5% dimethyl sulfoxide and cooled to the temperature of liquid nitrogen at a rate of ~1°/min. These frozen stocks of adult human skin fibroblasts were rapidly thawed in a 37° water bath, returned to tissue culture, and sent to the W. Alton Jones Cell Science Center where the colony size distributions were determined. Mass cultures were maintained in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate-buffered Eagle's basal medium with 10% fetal bovine serum (Rehatuin, Reheis Chemical Co., Kankakee, IL).

All clonal growth experiments were performed in MCDB 102 which was prepared according to the procedures of Ham *et al.* (9). Each freshly prepared batch of MCDB 102 was tested for its ability to support clonal growth of human diploid fibroblasts at between the 20th and 30th PD. Only those batches that gave results essentially equivalent to those reported previously (9) were used. The lot of fetal bovine serum used was prescreened for its ability to promote clonal growth of WI-38 cells in Eagle's basal medium.

Rapidly proliferating cultures were trypsinized prior to confluency. The cultures growing in 25-cm² flasks were rinsed once with 5 ml of buffered medium without serum and washed with 5 ml of 0.25% crude trypsin in Eagle's basal medium. All except 0.5 ml of the trypsin solution was removed and the cultures were incubated at 37° for approximately 10 min, until the cells were rounded and could be detached from the flask by a sharp tap. Then, 4.5 ml of MCDB 102 (9) supplemented with 10% fetal bovine serum was immediately added to the flask and a single-cell suspension prepared by gentle pipetting. The percentage of single cells was determined by hemocytometer count, and pipetting was continued until the suspension contained at least 98% single cells. Ten to 20 cells were plated into each of several 60-mm tissue culture dishes (Lux no. 5220) containing 5 ml of MCDB 102 with 10% fetal bovine serum. The clonal plates were incubated undisturbed for 14 days at 37° in a 5% CO_2/95% air atmosphere at 98–100% relative humidity. A 2-week incubation period was chosen to allow small colonies (200 cells or fewer) to reach their maximal size (i.e., the end of their proliferative potential) and thus reduce variation between experiments due to differences in clonal growth rate. At the end of the 2-week incubation period, the plates were fixed in 10% formalin, rinsed in water, and stained with 0.5% crystal violet.

Abbreviations: PD, population doubling(s); PDL, population doubling level; PDR, population doublings remaining; CSD, colony size distribution; CS, colony size.
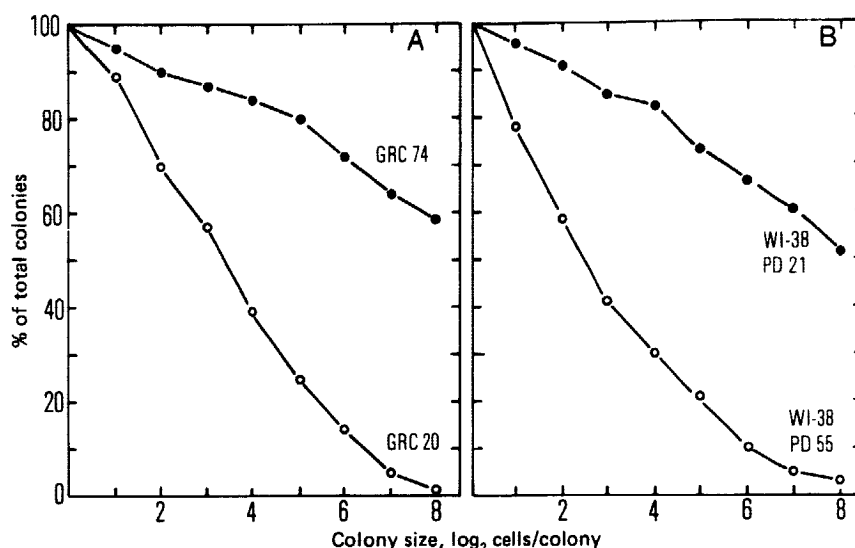
FIG. 1.   Percentage of colonies able to attain at least a specified size vs. colony size. Colony size is expressed as $\log_2$ of the number of cells per colony. (A) Adult human skin cultures from a young (GRC 74, age 33 years) and an old (GRC 20, age 80 years) donor were cloned at the 10th PD *in vitro*. (B) Human embryonic lung fibroblasts (WI-38) were cloned at the 21st and 55th PD *in vitro*.

The number of cells in each colony (defined as one or more attached cells) was determined (up to a maximum of 256 cells) with a dissecting microscope at ×20–×40 magnification. A total of 150–200 colonies were scored for each distribution. Colony size (CS) was designated by the number of doublings necessary for the initial single cell to reach the observed cell number. All studies on skin fibroblasts were performed on a double-blind basis and only after completion of these studies was the code broken.

RESULTS

Typical CSDs of cell lines obtained from skin fibroblast cultures from young and old donors cloned at PDL 10 are shown in Fig. 1A. For comparison, distributions for WI-38 cultures early (PD 21) and late (PD 55) in their *in vitro* life-span are shown in Fig. 1B. Distributions are presented as the percentage of colonies containing at least a specific number of cells 2 weeks after seeding. Approximately 50% of the cells inoculated in each case attached to the plate. Although the majority of cells cloned from the young donor culture (GRC 74) were able to produce colonies of 256 or more cells, indicating eight or more cell PDs, only 2% of cells cloned from the old donor line (GRC 20) were able to reach this state of proliferation. If one examines the percentage of cells able to yield colonies of eight or more cells, reflecting three or more cell PDs, even modest proliferative ability is impaired in the old donor culture when compared to the young donor culture (57% vs. 87%). The difference in CSDs between these skin fibroblast cultures from young and old donors after 10 PDs *in vitro* closely approximates the changes observed in the CSDs of fetal lung fibroblasts as they traverse their *in vitro* life-span.

Previous studies of CSDs of fetal lung fibroblast cultures (WI-38) have indicated that the percentage of colonies with 16 or more cells provided an excellent indicator of remaining *in vitro* life-span (8). Examination of cell cultures derived from a young (28 years) and an old (82 years) human donor at different PDs *in vitro* have confirmed this close relationship between PDs remaining (PDR) and CSD (Fig. 2). Comparison of the linear regression of percentage of colonies, from young and old donor cultures, with 16 or more cells as a function of PDR [PDR = 0.53(CS ≥ 16) + 0.6] reveals no significant differences.

The two-tailed 5% test (10) indicates that the slopes of the two regression lines are essentially the same. Both regression lines intercept the y axis at positions not significantly different from 0. It should also be noted that the linear regression for these adult skin fibroblasts is substantially the same as that for embryonic lung fibroblasts.

When the percentage of colonies with 16 or more cells is examined in these three cultures (young adult donor, old adult donor, WI-38) as a function of PDs completed *in vitro*, parallel regression lines are observed (Fig. 3). At the same level of *in vitro* PD, the embryonic cell line (WI-38) consistently provided the highest percentage of colonies with 16 or more cells and the old donor cell line had the lowest levels. Results from the young donor culture were intermediate. Because these regressions intercept the horizontal axis at a point approximating their total *in vitro* life-span, this type of plot appears to permit prediction of the *in vitro* life-span of human fibroblast cultures. The only requirement is that three or more points be taken early during *in vitro* cultivation.

To further assess the relationship between CSDs and *in vivo* human aging, CSs were measured on cell cultures from nine young donors and eight old donors under double-blind condi-
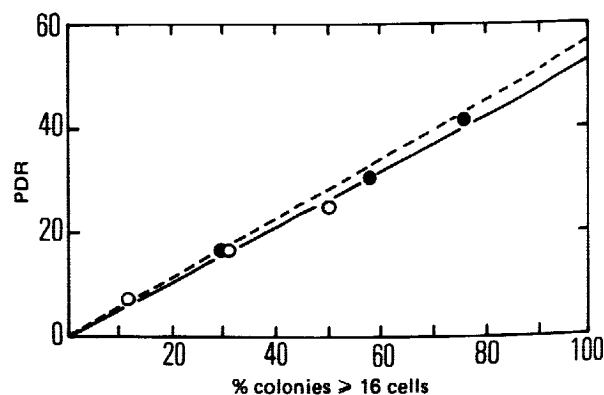

FIG. 2.   PDR vs. the percentage of colonies with 16 or more cells. The broken line is the linear regression for WI-38 cultures [PDR = 0.56(CS ≥ 16) + 0.6] (8). The solid line is the linear regression for adult skin cultures from a young (●) donor, GRC 72, and an old (○) donor, GRC 119.

Table 1. Percentage of young adult skin fibroblast colonies attaining X or more doublings

GRC	Donor age, yr	PD*	PDR†	1	2	3	4	5	6	7	8
25	31	13	36	90	80	73	69	65	60	50	41
68	27	13	34	88	72	64	54	48	38	26	14
75	29	11	36	89	78	71	66	63	55	45	36
104	29	15	34	86	73	64	60	55	52	48	46
74	33	10	43	95	90	87	84	80	72	64	59
66	28	8	41	88	73	67	63	52	40	25	7.1
64	33	9	34	89	75	70	67	62	51	37	25
110	33	5	50	90	85	84	82	80	76	69	60
72	28	5	42	96	83	78	76	73	71	68	65
$\bar{x}$	30.1			90.0	78.7	73.1	69.0	64.2	57.2	48.0	39.2

* PDs completed in culture at time of cloning.
† PDR in the *in vitro* life-span when the culture was cloned.

Table 2. Percentage of old skin fibroblast colonies attaining X or more doublings

GRC	Donor age, yr	PD*	PDR†	1	2	3	4	5	6	7	8
6	69	12	7	89	75	69	53	42	34	17	4.2
20	80	10	27	89	70	57	39	25	14	5	1.5
107	81	11	40	86	72	68	63	60	57	47	39.0
90	85	6	21	74	46	35	25	18	13	7	0.8
89	83	9	30	88	74	65	58	49	39	29	21.0
65	89	10	23	83	71	62	57	53	43	34	24.0
119	82	5	32	80	63	55	49	44	30	24	16.0
97	75	5	32	86	63	54	40	34	29	25	25.0
$\bar{x}$	80.5			84.4	66.8	58.1	48.0	40.6	32.4	23.5	16.4

* PDs completed in culture at time of cloning.
† PDR in the *in vitro* life-span when the culture was cloned.

tions (Tables 1 and 2). At all levels of CS, a difference was observed between the young and old donor cell cultures. In Fig. 4, the number of *in vitro* PDR of each of these 17 cultures is plotted as a function of percentage of colonies with 16 or more cells (CS $\geq$ 16). The CSD was determined only once for each culture (between the 5th and 15th PDs *in vitro*). The least squares linear regression derived from these data, PDR = 0.44(CS $\geq$ 16) + 6.8, has a correlation coefficient of 0.69 and is not significantly different (SD of PDR about the regression line, 7.3) from the regression of single-cell cultures sampled at several points of their *in vitro* life-span (Fig. 2). Thus, even a single determination of CSD may be able to predict remaining *in vitro* life-span.

In Fig. 5, *in vitro* life-spans and CSDs of young and old donor cultures are compared. Both parameters appear to correlate equally well with donor age and have relatively equal degrees of overlap and statistical significance. The CS $\geq$ 16 values for eight of the nine young donors were larger than those of seven of the eight old donors. Elementary combinatorics reveals the probability of this event by chance alone to be less than 0.001. The *in vitro* life-spans of all nine young donors were in excess

of all but one of those of the old donors, an equally improbable event by chance alone.

DISCUSSION

The proportion of cells unable to divide in clonal culture has been reported to increase exponentially as the PDL of the parent culture increases (11). A similar relationship has been reported for cells in mass cultures, with the uptake of [^{3}H]-thymidine used as an indication of ability of cells to divide, and it has been proposed as an index of cell culture aging (12). However, other investigators have reported a more complex relationship between the proportion of cells incorporating [^{3}H]thymidine and culture age (13). Theoretical studies have predicted that the distribution of CSs obtained from human fibroblast cultures would be a sensitive index of the number of *in vitro* PDR (*in vitro* cell culture "age") (14). Experimental studies on fetal lung fibroblast (WI-38) cultures have confirmed this prediction (7). The present study of adult human skin fibroblasts further supports this relationship between CSD and remaining culture life-span.

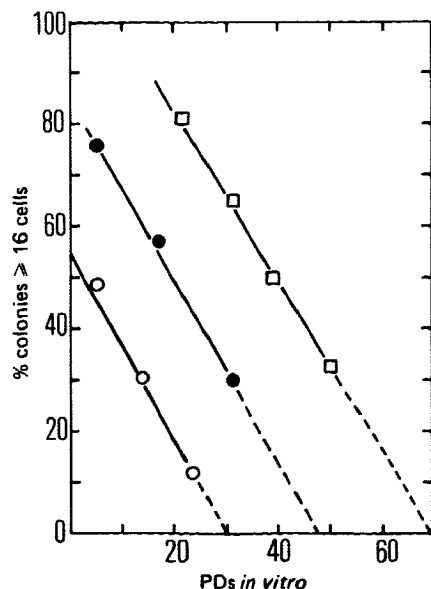Of particular interest is the relationship between CSD and

FIG. 3. Percentage of colonies with 16 or more cells vs. number of *in vitro* PDs completed. □, Embryonic lung cultures, WI-38; ●, cultures from young adult human skin, GRC 72; ○, cultures from old human skin, GRC 119.
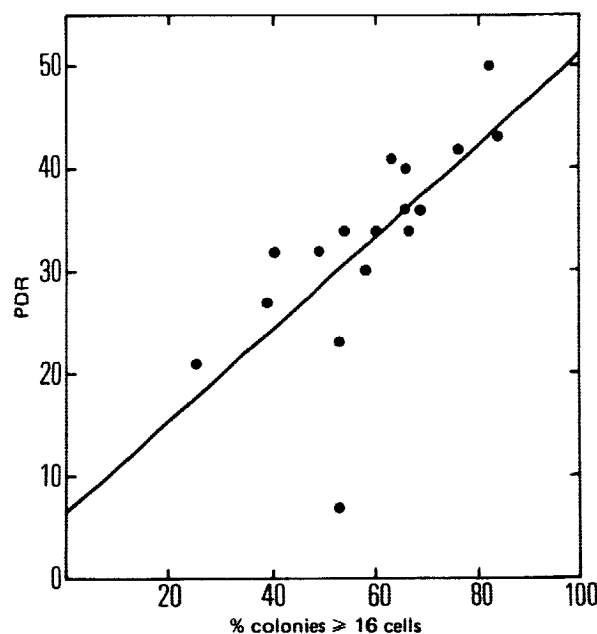
FIG. 4. PDR vs. percentage of colonies with 16 or more cells. Each point represents a culture from a different donor (nine young and eight old donors).
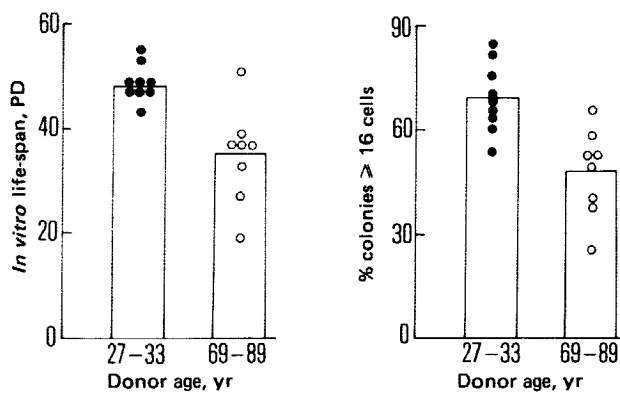
FIG. 5.   (*Left*) Total *in vitro* life-span of cultures isolated from young and old donor skin. (*Right*) Percentage of cells able to form colonies of 16 or more cells. Cells were isolated from the same cell lines shown in *Left*.

PD *completed*. In all three types of cell cultures examined (fetal lung and young and old adult skin fibroblasts), parallel linear regressions were generated. The displacement of these regressions appeared to be determined by the age of the donor. This would suggest that CSD may be an accurate measurement of the *in vivo* age of the donor from which the culture was established and may reflect the number of previous *in vivo* PDs.

Earlier investigations have indicated that the *in vitro* proliferative capacity (*in vitro* life-span) is inversely related to donor age (5–7). The results described above indicate that CSD appears to correlate equally well with the age of the cell culture donor. However, measurements of CSDs offer several distinct advantages over determinations of total *in vitro* life-spans. Perhaps the most appealing of these is the time involved in obtaining the CSD, 2 weeks compared to 6–9 months to determine the *in vitro* life-span of a culture. This is of particular importance if one would like to incorporate examination of *in vitro* proliferative studies into a comprehensive longitudinal study of aging. Another advantage of CSD determinations is that the reproducibility of the experimental conditions can be more effectively controlled. This can be done by maintaining a frozen stock of a standard cell line at a certain *in vitro* PDL in liquid nitrogen, so that it can be thawed and tested at regular intervals. In this way, the CSD can be standardized for tissue culture variables that can affect *in vitro* life-span, such as serum and medium quality. The point (CS $\geq$ 16 = 53, PDR = 7) in Fig. 4 was derived from a cell line that may have had an artificially shortened *in vitro* life-span due to suboptimal experimental conditions. This cell line, GRC 6, went from a rapidly proliferating state to an essentially nonproliferating state within two PDs compared to the more gradual decline, usually over about 10 PDs, observed for the other lines used in these experiments.

In these studies of CSD and in previous studies of *in vitro* life-span (5, 6), overlap was observed between old and young donor cultures. Factors that probably contribute to this observed overlap include: (*i*) normal genetic heterogeneity of human populations, (*ii*) use of chronological age as an indicator of biological age, (*iii*) selection of a vigorous old population, because less vigorous individuals may have died before age 60, and (*iv*) variation in explantation and culture techniques (6) and serum lots (E. L. Schneider, unpublished data).

Perhaps the most important of these factors is genetic heterogeneity. This is illustrated by comparing successive CSDs

obtained from serial subcultures of a specific cell strain (Fig. 2) with those obtained from different cell strains (Fig. 4). A significant increase in variance reflected by a decreased correlation coefficient is observed in the latter situation.

An additional possible source of variation in the data shown in Fig. 5 is that the CSDs were determined over a range of 5–15 *in vitro* PDs. The effect of this range of PDLs on the variation of CS within the two age groups was tested by using the regression equation obtained from Fig. 4 to estimate the CS $\geq$ 16 expected at PD 10 for each cell line. The percentage of colonies with 16 or more cells shown in Fig. 5 ranges from 84 to 54 (mean, 69) for the young donor group and from 63 to 25 (mean, 48) for the old donor group. After normalizing to PD 10 *in vitro*, the range was 84–58 (mean, 69) for the young donor group and 65–16 (mean, 45) for the old donor group. It therefore appears that CSDs are more sensitive to intrinsic properties of the cell cultures than to small differences in the *in vitro* PDL at which the cultures were sampled.

These studies, like most aging research, were cross-sectional in nature and are restricted by the inherent limitations of this approach. However, it is anticipated that a comprehensive longitudinal study may be even more informative. This could be accomplished by measuring CSDs of cultures from members of a longitudinal aging research program.

Because the CSD is a sensitive measure of the number of PDR in the *in vitro* life-span of a culture, it can be used to assess rapidly the effectiveness of experimental treatments that might increase or decrease the *in vitro* life-span of a culture. In a separate series of experiments (O. Pereira-Smith and J. R. Smith, unpublished data) it has been confirmed that the CSD accurately reflects the increase in life-span obtained by continuous growth of cultures in hydrocortisone (15, 16).

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